Effects of testosterone on cancellous bone, marrow adipocytes, and ovarian phenotype in a young female rat model of polycystic ovary syndrome

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Objective: To investigate the effects of testosterone on cancellous bone and marrow adipocytes in a young female rat model of polycystic ovary syndrome (PCOS).
Design: Comparative and controlled study.
Setting: University animal research laboratory.
Patient(s): Fifty-one Sprague-Dawley rats.
Intervention(s): The rats were divided into four groups based on the day of testosterone propionate (0.1 mg/weight (g)) injection: no testosterone treatment (control group, C); injected on the ninth day after birth (9D); injected 4 weeks after birth (4W); and injected 8 weeks after birth (8W). About 16 weeks after birth, all animals were killed.
Main Outcome Measure(s): Bone mineral density (BMD) and bone and fat histomorphometry for the proximal tibia and serum hormonal parameters were measured.
Result(s): The ovaries of group 9D showed many cystic follicles without corpora lutea. The BMD of group 9D (0.309 ± 0.023 g/cm^2) was significantly higher than the other groups groups (CONT, 0.262 ± 0.017; 4W, 0.256 ± 0.017; 8W, 0.256 ± 0.022 g/cm^2; $P<.0001$). Based on bone histomorphometry, group 9D had a higher bone volume (BV/TV), lower bone formation (OV/BV, OS/BS, sLS/BS, MAR, BFR/BS), lower bone resorption (ES/BS, Oc.S/BS), and lower rate of longitudinal growth compared to the other groups. Based on fat histomorphometry, group 9D had a lower bone fat volume and number of fat cells in the bone marrow compared to the other groups. On the other hand, groups 4W and 8W showed similar values of bone and fat histomorphometric parameters to group C.
Conclusion(s): Female rats receiving testosterone within nine days of birth develop polycystic ovaries, high bone volume, low bone turnover, and lower fat content in the bone marrow. (Fertil Steril® 2005;84(Suppl 2):1277–84. © 2005 by American Society for Reproductive Medicine.)

Key Words: Testosterone, bone histomorphometry, bone marrow adipocyte, polycystic ovary syndrome, rat

Osteoporosis in women is determined not only by aging and estrogen-dependent bone loss during menopause, but also by the peak skeletal mass attained and maintained in young adulthood (1, 2). There are several published articles involving inherited defects in men showing that the positive effects of androgens on bone are mediated through estrogenic action, but the effects of androgens on bone are unclear (3, 4). Although the influence of estrogens (both endogenous and exogenous) on bone metabolism in women has been examined extensively, the effects of androgenic hormones on bone mass in women are unclear. If the protective effect of estrogens on bone mass in women also extends to androgens, then androgenic effects during the adrenarche and the pubertal transition may be of particular importance in achieving maximum bone density in women.

Polycystic ovary syndrome (PCOS) is a common endocrine disorder characterized by anovulation, infertility, obesity, and hyperandrogenism in women of reproductive age, affecting 5% to 10% of premenopausal women (5). Insulin resistance and compensatory hyperinsulinemia are prominent features of PCOS (6). The relatively high prevalence of PCOS and its presentation early in life render this disorder of particular clinical importance. Furthermore, it is important to determine the effects of androgen, and potentially insulin therapy, on the attainment of maximal bone mass. It has been suggested by various studies that women with PCOS are protected from bone loss and that the obesity, hyperandrogenism, higher E_2 concentrations, and hyperinsulinemia associated with insulin resistance may be the protective factors against bone loss (7–10). However, PCOS is a complex disorder, and several components of the syndrome may be related to bone density through common metabolic mechanisms not necessarily mediated specifically by androgenic steroids.

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In this study, we investigated the effects of testosterone on cancellous bone and marrow adipocytes in a young female rat model of PCOS.

MATERIALS AND METHODS

Animals

Fifty-one Sprague-Dawley rats (Charles River Laboratory, Kanagawa, Japan) used in this study were kept in a room maintained at 25°C with a 12 h light/12 h dark cycle. A standard diet containing 24.9% protein source, 51.4% carbohydrate, 4.6% lipids, 3.7% fiber, 6.7% ash, and 8.6% moisture, containing 1.18% calcium (Ca), 1.09% phosphorus (P), 250 IU/100 g vitamin D3 (Clea, Tokyo, Japan) was made available ad libitum to all rats. The Ca and P content were higher than the recommended levels for pregnant, lactating, and nonpregnant rats, and the Ca/P ratio was >1.0, fulfilling what appears to be the requirements for soft tissue calcification (11). All rats were injected subcutaneously with testosterone propionate (0.1 mg in 0.004 mL olive oil/weight (g)) or olive oil only (12, 13). The rats were divided into four groups based on the day of injection: C: 13 rats injected with olive oil only on the ninth day after birth (control group); 9D: 14 rats injected on the ninth day after birth; 4W: 12 rats injected 4 weeks after birth; and 8W: 12 rats injected 8 weeks after birth.

About 16 weeks after birth, blood was sampled from the heart and the animals were killed under ether anesthesia. Body weight was measured before killing them. After killing, the ovaries and abdominal fat tissue were removed from each rat and weighed. The intraabdominal fat tissue was removed from the retroperitoneal, the mesenteric, and the greater omental adipose tissue completely via an abdominal approach. The right tibia was removed and fixed in 70% ethanol for at least 2 weeks. Both ovaries were fixed with 20% formalin, paraffin embedded, serially sectioned, and stained with hematoxylin and eosin. The collected blood was centrifuged, and the sera was frozen at −20°C until the time of assay. The animals were maintained in accordance with the guiding principles in the "Care and Use of Animals" of the American Journal of Physiology.

Bone Mineral Density (BMD)

The BMD of the proximal head of the right tibial bone was measured by ultrahigh-resolution-mode dual-energy x-ray absorptiometry (DXA) with a QDR-2000 system (Hologic, Waltham, MA). The coefficient of variation for the DXA measurement was 2.3%.

Bone and Fat Histomorphometry

Each rat was injected subcutaneously with tetracycline hydrochloride (Sigma Chemical Co., St. Louis, MO) 20 mg/kg 6 days and 1 day before killing. After killing, the right tibia was removed from each rat, trimmed to remove the muscle, fixed with 70% ethanol for at least 2 weeks, then stained with Villanueva bone stain (Maruto Instrument Co., Tokyo, Japan) for 10 days, dehydrated with ethanol, and embedded in methyl methacrylate (Wako Chemicals, Kanagawa, Japan) without decalcification. Frontal specimens of the proximal part of each tibia were cut and 5-μm-thick parasagittal sections were cut using a Junk-K Microtome (Carl Zeiss,

TABLE 1

Weight and serum hormonal and biochemical data.

		C, 13	9D, 14	4W, 12	8W, 12
Body weight	g	289.0 ± 28.9	298.9 ± 22.3[c]	310.8 ± 27.0[c,d]	274.4 ± 18.5
Ovary weight	g	0.15 ± 0.04	0.13 ± 0.03	0.14 ± 0.01[c]	0.11 ± 0.02[d]
Intraabdominal fat (total)	g	12.8 ± 4.1	12.7 ± 2.4[c]	14.0 ± 3.2	10.0 ± 3.4
Retroperitoneal cavity	g	8.5 ± 3.3	7.9 ± 1.9	9.3 ± 2.5	6.9 ± 2.6
Mesentery	g	3.1 ± 0.9	3.5 ± 0.8[c]	3.6 ± 1.1	2.2 ± 0.7
Omentum	g	1.3 ± 0.2	1.4 ± 0.4[b,c]	1.1 ± 0.4	1.0 ± 0.2[d]
Luteinizing hormone	ng/mL	4.4 ± 2.5	4.2 ± 1.7[c]	5.9 ± 3.1	8.5 ± 3.8[d]
Follicle stimulating hormone	ng/mL	45.0 ± 15.5	24.6 ± 14.7	27.8 ± 15.0	33.9 ± 3.1
Estradiol	pg/mL	23.5 ± 15.2	36.9 ± 16.4	25.6 ± 16.8	22.1 ± 10.6
Testosterone	ng/mL	0.031 ± 0.075	0.015 ± 0.055	0.08 ± 0.092	0.062 ± 0.082
Free-testosterone	pg/mL	0.14 ± 0.15	0.15 ± 0.20	0.29 ± 0.41	0.20 ± 0.26
Alkaline phosphatase	IU/L	285.1 ± 102.0	262.2 ± 113.2	342.5 ± 37.4	308.4 ± 152.7
Calcium	mg/dL	8.6 ± 2.7	8.4 ± 2.5	9.8 ± 0.3	9.8 ± 0.7
Phosphate	mg/dL	6.5 ± 2.8	7.2 ± 3.0	7.4 ± 0.4	6.0 ± 0.8

[b] Significantly different from group 4W, $P<.05$.
[c] Significantly different from group 8W, $P<.05$.
[d] Significantly different from group C, $P<.05$.

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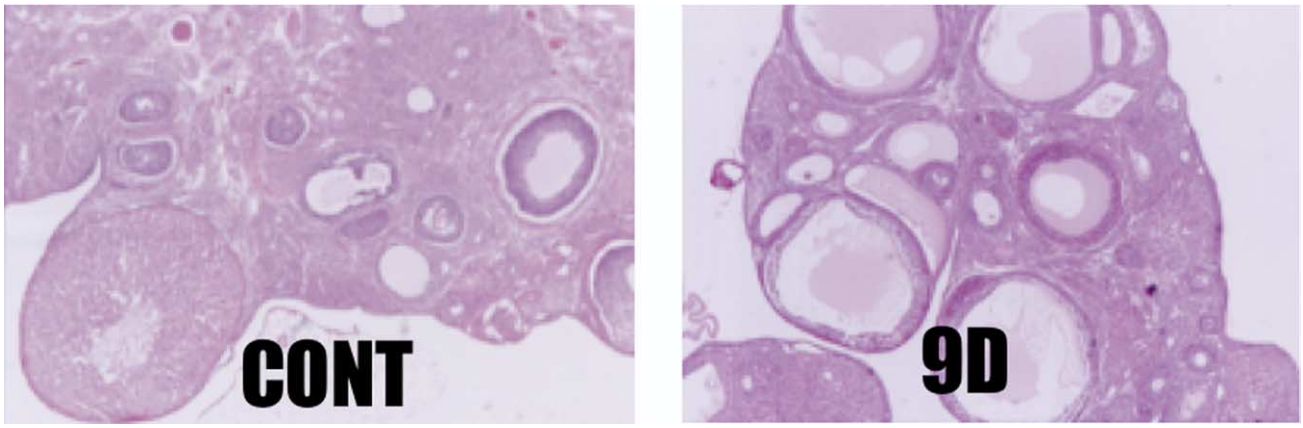
FIGURE 1

Histology of the ovary (×5). CONT: No testosterone treatment; 9D: testosterone treatment on the ninth day after birth.



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Heidelberg, Germany). The cancellous bone area of the proximal metaphysis was measured by a rectangular region (mean dimensions 2.11 ± 0.18 mm^2) in the secondary spongiosa located 1.0–1.8 mm from the growth cartilage–metaphyseal junction and 160 μm from the endocortical surface. Bone histomorphometric measurements of the tibia were made using a semiautomatic image analyzing system (Bone Histomorphometry, version 1.5; System Supply, Nagano, Japan) and a fluorescent microscope (Optiphot; Nikon, Tokyo, Japan) set at a magnification of 320×.

Bone histomorphometric parameters for the proximal metaphysis of the tibia were measured as described in the report of the ASBMR Histomophometry Nomenclature Committee (14). The growth plate width (GPWi) and the longitudinal growth rate (LGR) represent dynamic histomorphometric parameters of the growing long bone metaphysis (15). In addition, fat histomorphometric parameters for the tibia were measured. The fat volume (Fa.V) is expressed as a percentage of the marrow volume (Ma.V) and calculated as the percent fat volume (Fa.V/Ma.V, %). The number of fat cells (N.Fa) per unit area (mm^2) of the marrow was determined manually and calculated as the fat cell number (N.Fa/Ma.V, number of cells/mm^2), and the volume of each fat cell was determined and calculated as the unit fat volume (Fa.V/N.Fa, μm^2) (16).

Serum Biochemical Studies

Serum LH and FSH were measured by enzyme immunoassay (EIA) using the rat LH EIA system and the rat FSH EIA system, respectively (Amersham Pharmacia Biotech, Piscataway, NJ). Serum estradiol (E_2), testosterone (T), and free-testosterone (free-T), were measured by radioimmunoassay at Mitsubishi Kagaku Bio-Clinical Labs. Serum calcium was measured by o-CPC method (Serotec Co., Sapporo, Japan), phosphate by enzyme method (Kyowa Medex Co., Tokyo, Japan), and alkaline phosphatase (ALP) by JSCC standardization corresponding method (Wako Co., Tokyo, Japan) in our hospital laboratory.

Statistics

Data are reported as mean ± SD. All data management and statistical computations were performed using Stat View 4.0 (Abacus Concepts, Berkeley, CA). The statistically significant differences among the four groups were evaluated by one-way factorial-measures ANOVA followed by Fisher's least significant difference method for multiple comparisons. A value of $P<.05$ was considered significant.

RESULTS

Weight of Body, Ovary, and Abdominal Fat

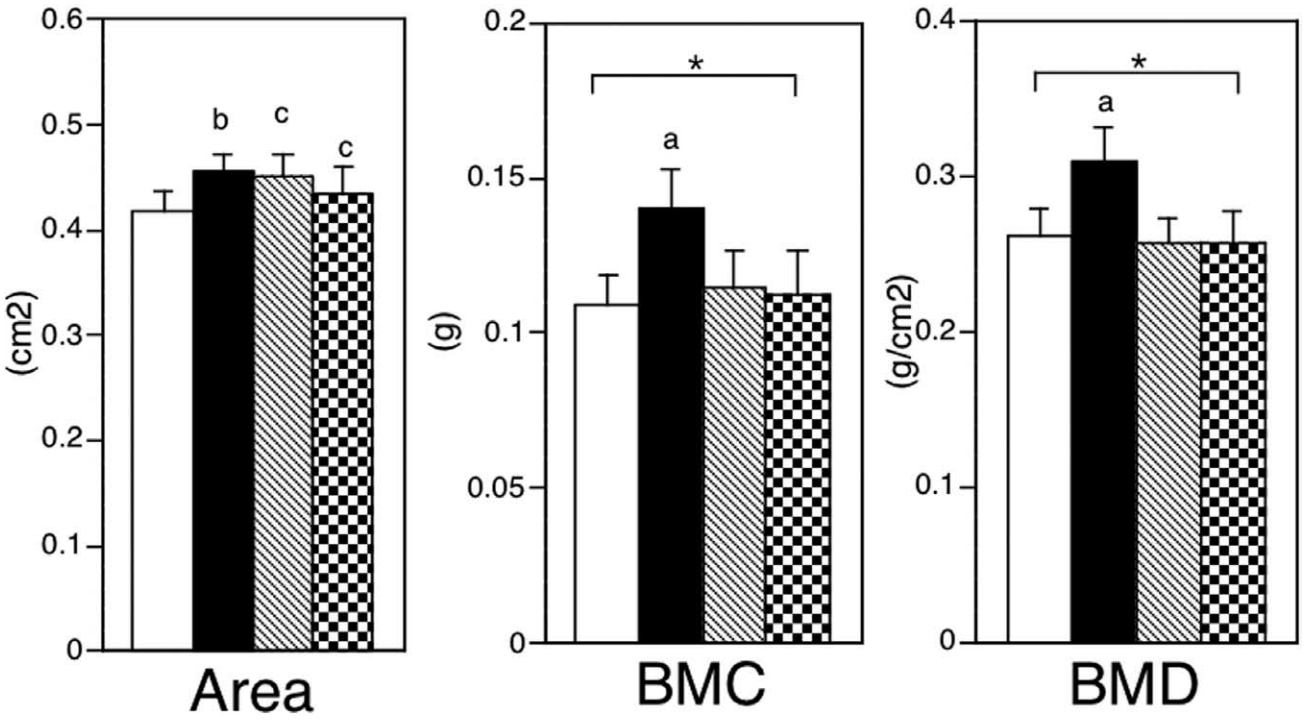
The body weight of group 8W was the lowest in the four groups and significantly lower than that of groups 9D and 4W, although it was not significantly different from the control group. The body weight of group 4W was highest and significantly higher than the control group. The ovarian weight of group 8W was the lowest of the four groups and significantly different from groups 4W and C. The intraabdominal fat weight of group 8W was the lowest of the four groups and was significantly different from the other groups. When we divided the intraabdominal fat into three regions (retroperitoneal cavity, mesentery, and greater omentum), the values of mesentery and omentum for group 8W were the lowest (Table 1).

Histology of the Ovaries

The normal ovaries from group C contained some follicles at various stages of development and several generations of

FIGURE 2

Area, bone mineral content (BMC), and bone mineral density (BMD) of the proximal tibia measured by dual energy x-ray absorptiometry. White bar: no testosterone treatment (C); black bar: testosterone treatment on the ninth day after birth (9D); shaded bar: testosterone treatment 4 weeks after birth (4W); checkered bar: testosterone treatment 8 weeks after birth (8W). One-way factorial-measures ANOVA: *$P<.0001$. Fisher's least significant difference: a: $P<.05$ vs. C, 4W, and 8W; b: $P<.05$ vs. C and 8W; c: $P<.05$ vs. C.



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corpora lutea. In contrast, the corpora lutea were absent and there were many cystic follicles in group 9D. The ovaries of groups 4W and 8W were not polycystic (Fig. 1).

Serum Hormone Concentrations and Bone Parameters

The serum LH concentration in group 9D was similar to the value for the control group. The LH concentration in group 8W was significantly higher than groups 9D and C. The serum E_2 concentration in group 9D tended to be higher than in the other groups, although there were no significant differences among the four groups by ANOVA ($P=.06$). There were no significant differences among the four groups with respect to serum T and free-T concentrations. The values for serum ALP, calcium, and phosphate were similar in all four groups (Table 1).

BMD of the Proximal Tibia

The bone areas of groups 9D, 4W, and 8W (0.455 ± 0.018, 0.452 ± 0.021, and 0.435 ± 0.026 cm^2, respectively) were slightly higher than in the control group (0.418 ± 0.019 cm^2). The tibial bone mineral content (BMC) value of group 9D (0.140 ± 0.013 g) was significantly higher than the other groups (C, 0.109 ± 0.010; 4W, 0.115 ± 0.012; 8W, 0.112 ± 0.015 g; $P<.0001$). The value for the proximal tibial BMD of group 9D (0.309 ± 0.023 g/cm^2) was also significantly higher than the other groups (C, 0.262 ± 0.017; 4W, 0.256 ± 0.017; 8W, 0.256 ± 0.022 g/cm^2; $P<.0001$) (Fig. 2).

Bone Histomorphometry

Structural parameters. The BV/TV and the Tb.N in group 9D were significantly higher than in the other groups. The Tb.Th was higher in group 9D than in group 4W or C, but there was no significant difference between groups 9D and 8W (Table 2 and Fig. 3).

Cellular activity parameters/formation parameters. All formation parameters in group 9D were significantly lower than in the other groups, except for the dLS/BS, which was similar in groups 9D and 4W.

Cellular activity parameters/resorption parameters. The ES/BS and the Oc.S/BS in group 9D were significantly lower than in the other groups, and those in group 4W were also significantly lower than in group C, although they were not

TABLE 2

Trabecular bone histomorphometry of the tibial proximal metaphysis.

			C, 13	9D, 14	4W, 12	8W, 12
Bone volume	BV/TV	%	30.1 ± 6.0	52.8 ± 10.9[a]	35.9 ± 6.9	31.2 ± 5.9
Trabecular thickness	Tb. Th	μm	63.0 ± 7.5	76.0 ± 9.8[b,d]	65.8 ± 6.8	69.8 ± 7.5[d]
Trabecular number	Tb.N	n/mm	4.9 ± 0.9	6.9 ± 1.0[a]	5.4 ± 0.8[c]	4.5 ± 0.8
Osteoid volume	OV/BV	%	0.81 ± 0.53	0.11 ± 0.09[a]	0.83 ± 0.75	1.03 ± 0.85
Osteoid surface	OS/BS	%	10.4 ± 6.2	3.0 ± 2.0[a]	9.1 ± 7.3	10.7 ± 8.0
Single labeled surface	sLS/BS	%	10.3 ± 2.9	3.3 ± 1.8[a]	11.0 ± 5.1	10.9 ± 4.6
Double labeled surface	dLS/BS	%	11.5 ± 4.8	2.6 ± 1.3[c,d]	6.3 ± 5.2[d]	8.2 ± 5.8
Mineral apposition rate	MAR	μm/day	1.0 ± 0.1	0.7 ± 0.2[a]	1.1 ± 0.2[c]	1.3 ± 0.1[d]
Bone formation rate	BFR/BS	mm^3/mm^2/year	0.06 ± 0.02	0.01 ± 0.01[a]	0.05 ± 0.04	0.07 ± 0.04
Eroded surface	ES/BS	%	18.1 ± 5.2	8.5 ± 4.6[a]	13.4 ± 2.4[d]	15.2 ± 4.8
Osteoclast surface	Oc. S/BS	%	8.0 ± 2.9	3.7 ± 2.7[c,a]	5.5 ± 1.0[d]	6.6 ± 2.5
Growth plate width	GPWi	μm	132.0 ± 12.1	105.7 ± 11.4[d]	106.5 ± 9.7[d]	103.6 ± 10.7[d]
Longitudinal growth rate	LGR	μm/day	30.6 ± 3.6	23.5 ± 3.6[c,d]	23.6 ± 2.0[c,d]	32.0 ± 2.1

[a] Significantly different from every other group, $P<.05$.
[b] Significantly different from group 4W, $P<.05$.
[c] Significantly different from group 8W, $P<.05$.
[d] Significantly different from group C, $P<.05$.

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significantly different between groups 8W and C. Resorption parameters tended to be lower with earlier times of testosterone injection.

Dynamic histomorphometric parameters of the growing long bone metaphysis. The GPWi of all of the groups that received testosterone were significantly lower than in the control group. The LGR of groups 9D and 4W were significantly lower than in groups 8W and C.

Fat Histomorphometry

The Fa.V/Ma.V, N.Fa/Ma.V, and Fa.V/N.Fa in group 9D were significantly lower than in the other groups. There were no differences in the values between groups 4W, 8W, and C (Table 3, Fig. 4).

DISCUSSION

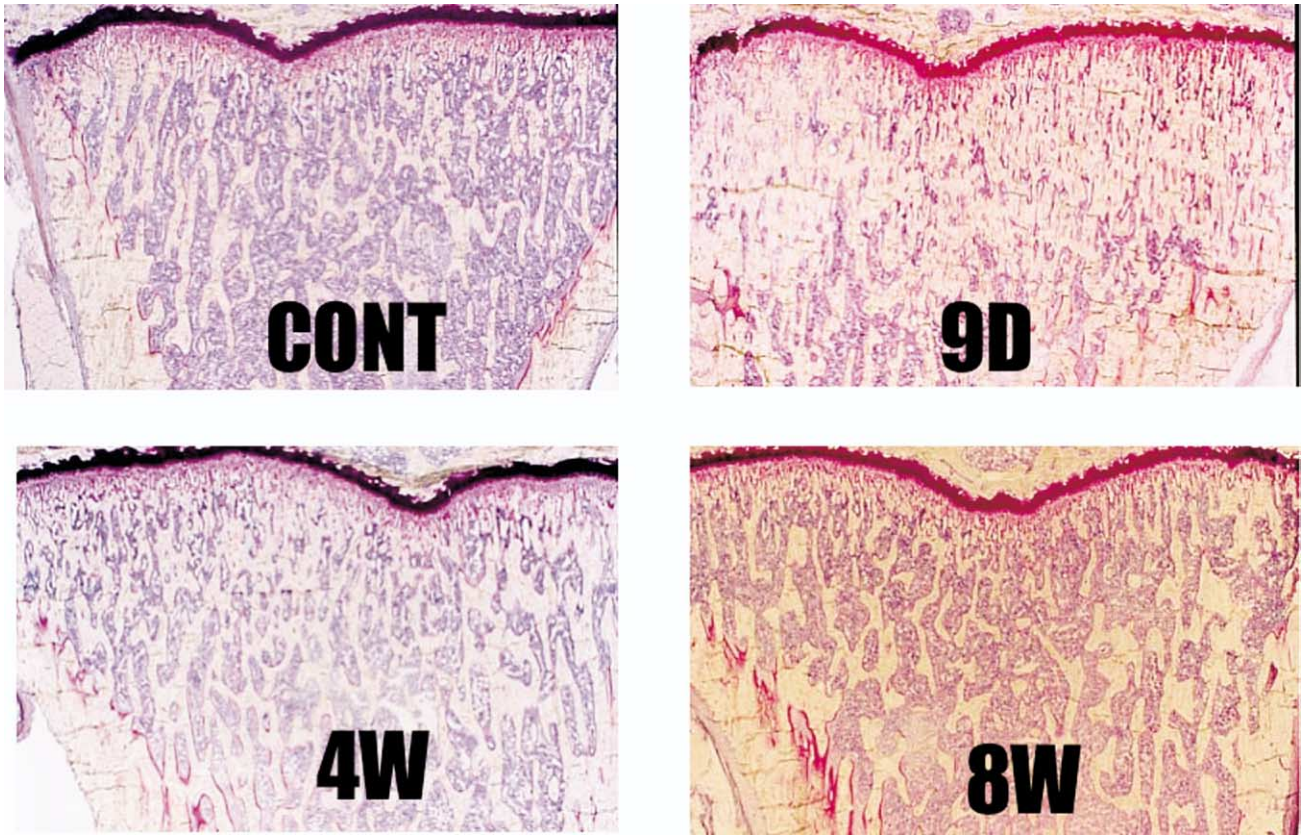
This study is the first to show that testosterone administration on the ninth day of life causes higher bone volume and lower bone turnover compared to control female rats. Cancellous bone loss in female ovariectomized rats can be inhibited not only by estrogen, but also by androstenedione and androgenic progestins (17, 18). Similarly, administration of antiandrogenic compounds has been shown to reduce bone mass in male and female rats (19). In this study, early administration of testosterone also causes polycystic ovaries. In human females seeking gender reassignment, long-term androgen (testosterone) treatment prior to surgery induces polycystic changes in the ovaries (20). Patients with PCOS have spine and femoral neck BMD values that are positively correlated with serum androstenedione and free testosterone concentrations, suggesting that in patients with PCOS the deleterious effects of amenorrhea on bone metabolism may be balanced by androgen overproduction (10).

The chronic elevation in the testosterone concentration may exert a positive influence on bone metabolism in women with PCOS, either directly through androgen receptors (ARs) present on bone-related cells or indirectly after conversion to E_2 in peripheral tissues. Although ARs are found primarily within the nucleus of all three types of bone cells (osteoblasts, osteoclasts, and osteocytes), ARs are mainly expressed in osteoblasts and to a greater degree in cortical rather than in cancellous bone (21). In a study of two prepubertal and eight pubertal girls with complete androgen insensitivity syndrome, BMD was significantly lower than in normal individuals (22), suggesting a role for androgens in bone mineral accrual. In a clinical study of surgically menopausal women, the combined estrogen and androgen therapy increased spine and hip BMD significantly more than the estrogen-alone therapy (23).

Our data show that the parameters of bone formation are lower, although the bone mass in the early testosterone group

FIGURE 3

Bone histomorphometry of the proximal metaphysis of the tibia (×20). CONT: No testosterone treatment; 9D: testosterone treatment on the ninth day after birth; 4W: testosterone treatment 4 weeks after birth; 8W: testosterone treatment 8 weeks after birth.



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was higher than the other groups. The growth plate width and the longitudinal growth rate of the group receiving early testosterone treatment were suppressed compared to other groups in this study. One of the reasons for this finding is that the animals were killed about 15 weeks after testosterone was administered, so the effect of testosterone may have dissipated, although testosterone may have accelerated bone formation immediately after testosterone administration. Recent studies showed that AR-mediated testosterone action is essential for periosteal bone formation and contributes to cancellous bone maintenance in male mice (24), and that serum testosterone increases bone formation and serum E_2 suppresses both bone formation and resorption in young men (25). However, our study may be the effect of acute increase of testosterone, and the blood testosterone levels of group D9 is the lowest at 16 weeks. Another reason of this finding may

TABLE 3

Fat histomorphometry of the tibial proximal metaphysis.

			C, 13	9D, 14	4W, 12	8W, 12
Percent fat volume	Fa. V/Ma. V	%	2.2 ± 1.2	0.3 ± 0.3[a]	1.8 ± 2.0	2.6 ± 2.9
Fat cell number	N. Fa/Ma. V	n/mm^2	55.0 ± 30.5	8.9 ± 10.5[a]	44.6 ± 44.6	64.3 ± 64.8
Unit fat volume	Fa. V/N. Fa	μm^2	403.9 ± 57.9	311.0 ± 98.6[a]	386.7 ± 38.2	395.3 ± 64.3

[a] Significantly different from every other group, $P<.05$.

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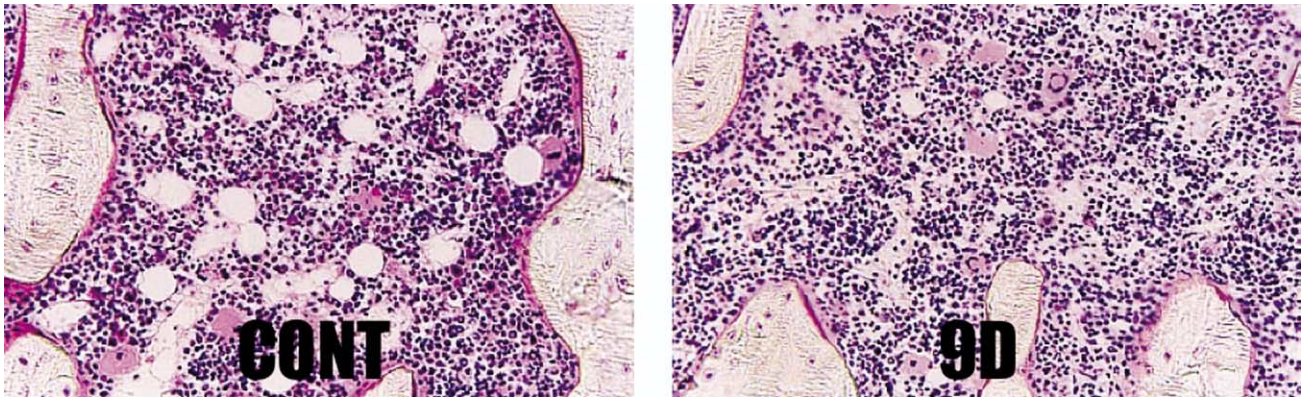
FIGURE 4

Fat histomorphometry of the proximal metaphysis of the tibia (×100). CONT: No testosterone treatment; 9D: testosterone treatment on the ninth day after birth.



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be that estrogen, which may be generated from testosterone, had suppressed bone turnover at the time of killing. The conversion of androgens to estrogens in the ovaries and in extraglandular tissue, with subsequent binding to estrogen receptors in target organs, is recognized as a primary indirect mechanism of androgen action on bone metabolism (26). Although there is no significant difference, group 9D has hyperestrogenic (not hyperandrogenic) tendency compared with other groups, and the bone phenotype would appear to follow from that, having nothing to do with androgens.

The second interesting result of this study was that testosterone was effective for enhancing bone metabolism only in young rats at nine days of age. Previous studies showed that polycystic ovaries and ovulatory failure in rats could be induced by the administration of testosterone propionate during the first 5 days of life in newborn female rats (27). It was also suggested that an "androgen-sensitive period" exists between birth and ten days of age. The higher bone mineral content observed in prepubertal girls with premature adrenarche reflects the presence of increased concentrations of androgens compared with normal individuals (28). A recent study demonstrated a significant increase in androgen concentrations during pregnancy in women with POCS (29). Transient intrauterine androgen exposure may cause disordered LH release, increased central adiposity, and defects in insulin secretion that are not manifested until puberty (30, 31). Accordingly, it is possible that androgen concentrations in utero and/or prepuberty could provide a potential source of androgen excess for the fetus or the child and contribute to the PCOS phenotype.

The third interesting result of this study was that the adipocyte volume and number in group 9D, which had a high BMD, significantly decreased compared to the other groups. We found inverse correlations between the cancellous bone volume and adipocyte volume and number and a reciprocal relationship between marrow adipocyte differentiation and bone remodeling in tibial metaphysis that are in keeping with previous studies (32–35). Other researchers have shown that the tibial proximal metaphysis loses cancellous bone more rapidly than the vertebral bodies (36) and that the number of marrow adipocytes in the metaphysis, but not the epiphysis, increases after the bone volume begins to decline (37). Stromal preadipocytes create a microenvironment conducive to osteoclast formation and resorptive activity through direct cell contacts and the release of soluble factors (38, 39).

We conclude that female rats receiving testosterone within nine days of birth develop polycystic ovaries, high bone volume, low bone turnover, and lower fat content in the bone marrow.

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